AWARD NUMBER: W81XWH-13-1-0361

TITLE: A Novel Tumor Antigen and Foxp3 Dual-Targeting Tumor Cell Vaccine Enhances the Immunotherapy in a Murine Model of Renal Cell Carcinoma

PRINCIPAL INVESTIGATOR: Li Shen Ph.D.

CONTRACTING ORGANIZATION:
Health Research, Inc.
Buffalo, NY14263

REPORT DATE: October 2014

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2014	Annual	15Sep2013-14Sep2014

4. TITLE AND SUBTITLE

A Novel Tumor Antigen and Foxp3 Dual-Targeting Tumor Cell Vaccine Enhances the Immunotherapy in a Murine Model of Renal Cell Carcinoma

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0361

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Li Shen

E-Mail: Li.Shen@roswellpark.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Health Research Incorporated,
Roswell Park Cancer Institute
Buffalo, New York 14263-0001

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

During the past year I have generated Foxp3-over expressing RENCA cells, as the source of candidate dual targeting tumor cells vaccine. We have performed controlled vaccine therapy in RENCA model in three different schedules. When applied in a pre-vaccine schedule, RENCA and RENCA Foxp3 tumor cell vaccine efficiently prevented or inhibited orthotopic tumor growth. In an intervention schedule setting, RENCA Foxp3 tumor cell vaccine was superior to the RENCA tumor cell vaccine, and significantly reduced tumor growth, as compared to the vehicle and RENCA vaccine treated groups. In addition, the RENCA Foxp3 vaccine had activity to reduce number of T regulatory cells (Tregs) in tumor infiltrates and reduce Foxp3 expression level in Tregs. Our result support testing of tumor cell vaccine for Renal cell carcinoma in a clinical settings and also suggest the tumor and Tregs dual targeting vaccine may have better success rate in patients with established disease.

15. SUBJECT TERMS
Renal Cell Carcinoma, vaccine therapy, regulatory T cells, myeloid derived suppressor cells, Tumor associated macrophages

16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	15	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Introduction:

Targeted therapies have shown clinical benefit in kidney cancer patients but the duration of the benefit is limited as most develop therapy resistance over time. Renal cell carcinoma (RCC) is the most common type of kidney cancer and a relatively more immunogenic cancer, as compared to other types of cancer. Therefore, immunotherapy such as cytokine and vaccine therapy represents an interest for treatment of RCC. A major barrier in vaccine therapy is represented by the presence of immunosuppressive factors predominant in cancer patients, such as regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs). Here we report a tumor cell vaccine designed to target Tregs while inducing immune response against tumor cells. During the past year I have generated Foxp3-over expressing RENCA cells, as the source of candidate dual targeting tumor cells vaccine. We have performed controlled vaccine therapy in RENCA model in three different schedules. When applied in a pre-vaccine schedule, RENCA and RENCA Foxp3 tumor cell vaccine efficiently prevented or inhibited orthotopic tumor growth. In an intervention schedule setting, RENCA Foxp3 tumor cell vaccine was superior to the RENCA tumor cell vaccine, and significantly reduced tumor growth, as compared to the vehicle and RENCA vaccine treated groups. In addition, the RENCA Foxp3 vaccine had activity to reduce number of T regulatory cells (Tregs) in tumor infiltrates and reduce Foxp3 expression level in Tregs. Our result support testing of tumor cell vaccine for Renal cell carcinoma in a clinical settings and also suggest the tumor and Tregs dual targeting vaccine may have better success rate in patients with established disease.

Keywords:

Renal Cell carcinoma
Tumor Cell Vaccine
Regulatory T cells
Myeloid Derived Suppressor Cells
Tumor Associated Macrophage
Tumor Microenvironment

Accomplishments:

The major goals of the project during the first year period are to determine the anti-tumor activity of the dual-targeting tumor cell vaccine in a murine RCC model, RENCA, and to evaluate the immuno-modulatory activity of the vaccine in the model. These goals during the first year award period are listed as part of the specific Aim1 and specific Aim3. Here I summarize our activities and accomplishment as listed under the Specific Aims and Tasks.

Specific Aim 1: To determine the anti-tumor activity of the dual-targeting tumor cell vaccine in a murine orthotopic RCC model, RENCA. (Month 1-14)

Task 1: Generate a RENCA Foxp3 cell line. (Month 1-4)

The MIGR-mFoxP3 vector containing mouse Foxp3 expressing construct was purchased from Addgene. The plasmid was amplified and checked to confirm its structure. Retroviral vector was produced by transfecting the plasmid in a retroviral package cell line.

The resulted retroviral vectors were used to infected RENCA cells, followed by multiple rounds (1 week, two weeks, three weeks and two months after infection) of flow cytometry sorting to select RENCA cells with stable incorporation of the MIGR-mFoxP3 construct, as the MIGR-mFoxP3 plasmid has a GFP marker gene (Figure 1A).

Finally, immunofluorescent staining of Foxp3 were performed to confirm that the infected and selected RENCA cells have Foxp3 over-expression (Figure 1B), and resulted in the RENCA Foxp3 cells, which were used to produce the tumor cell and Tregs dual targeting tumor cell vaccine.

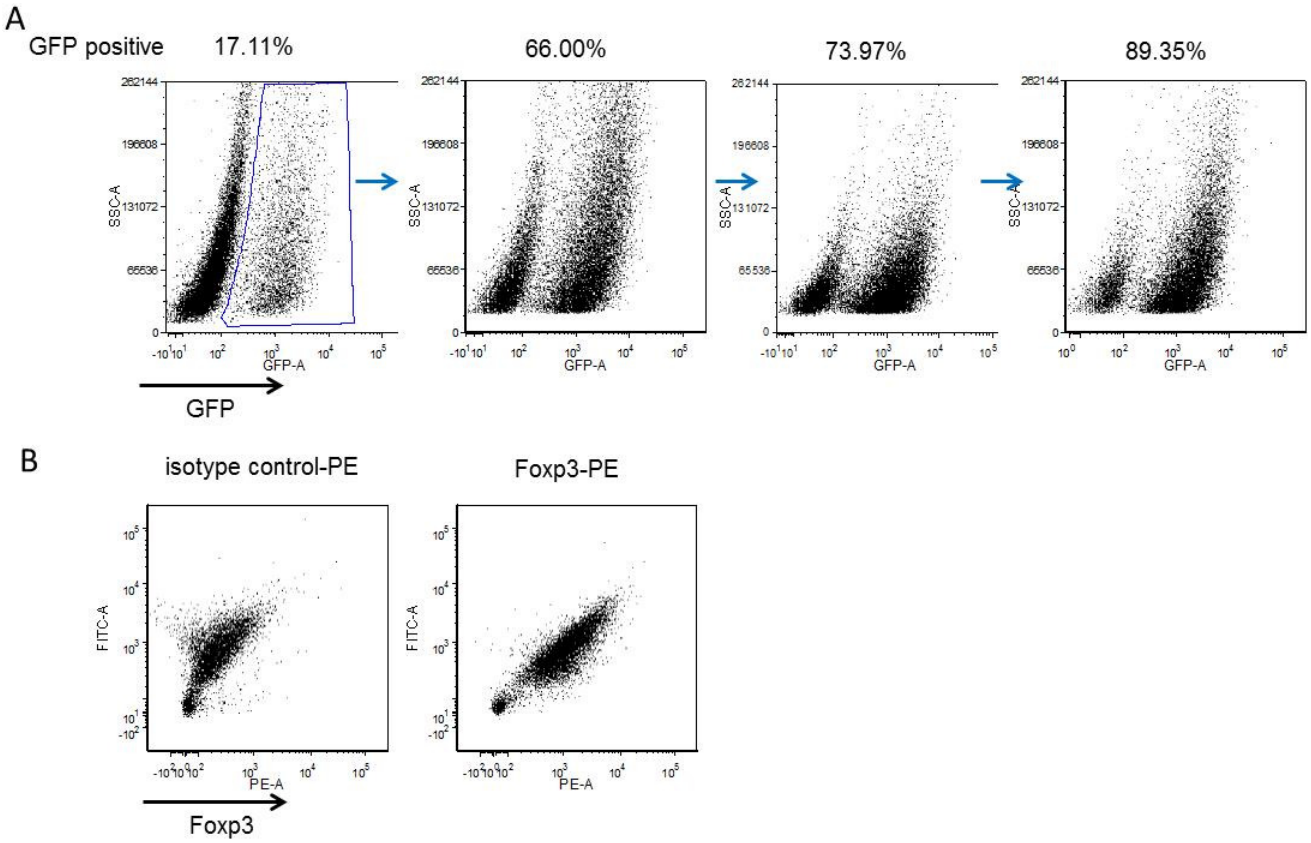


Figure 1: A. panels show the progress of selection of RENCA cells with integration of MIGR-Foxp3 construct

containing GFP selection marker. Numbers above plots show percentage of GFP positive cells. B. immuno-fluorescence staining show specific Foxp3 expression in RENCA Foxp3 cells, as compared to isotype control staining.

Task 2: Vaccine generation. (Month 5-10)

Tumor cell lysate vaccine has been prepared from RENCA Foxp3 cells by sonication of cells in PBS without carrier protein. Then the lysates were mixed with Montanide® and injected with GM-CSF. Each dose of vaccine is lysate from 1 x 10^6 cells. The dendritic cell (DC) vaccine has been produced by isolation, induction and pulse of mouse bone marrow cells with the suggested protocol from a collaborator. Each dose includes 1 x 10^6 DCs.

Task 3: Conduct therapy experiment to test the RENCA Foxp3 cell derived vaccines in the orthotopic RENCA models. (Month 5-10)

Task 4: Correlated studies. (Month 5-12)

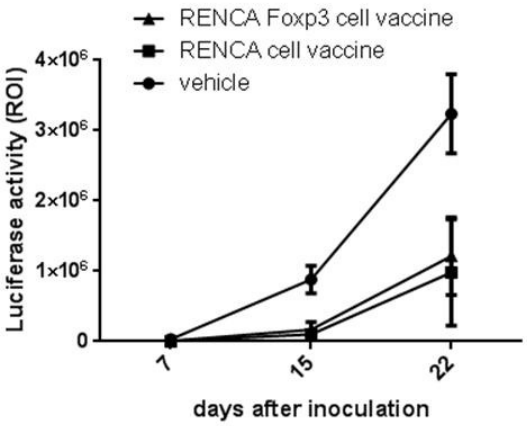
Task 5: Data collection and statistical analysis. (Month 10-12)

Majority of task 4 was in parallel with Task 5. The end part of Task 4 and the Task 5 will be data analysis of experiments in Task3. Therefore, the accomplishment from these tasks is summarized together here.

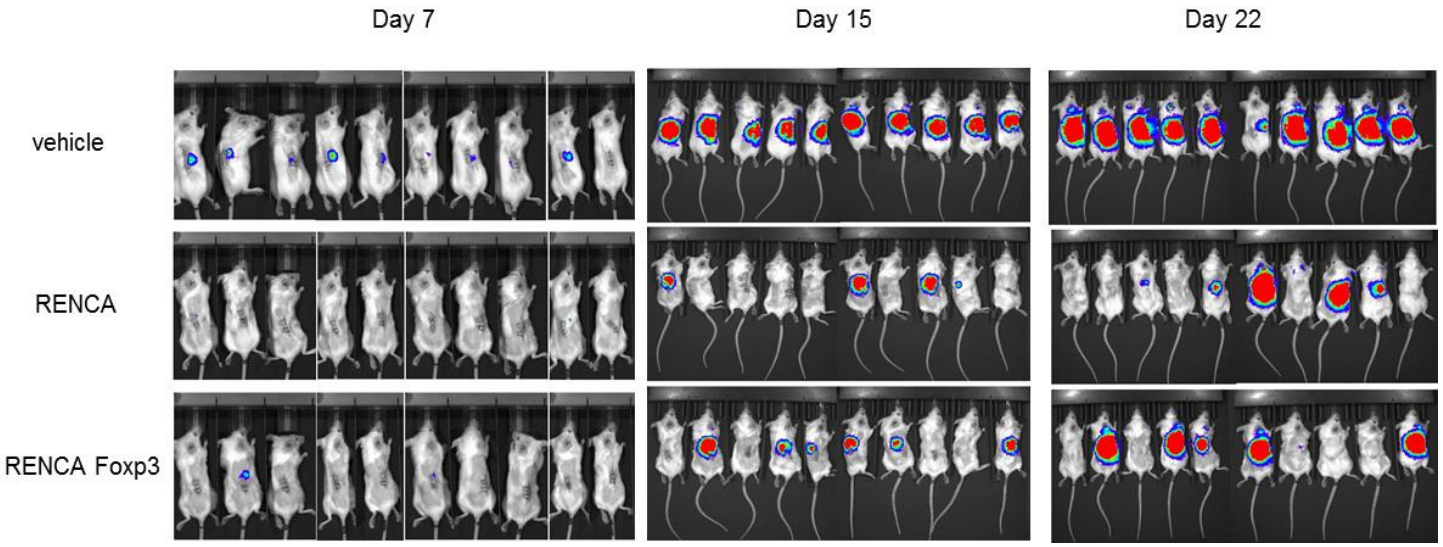
In order to test the RENCA Foxp3 cell vaccine, the therapy study has been conducted in three different vaccine schedules. The first schedule is pre-vaccine schedule, in which the mice were randomly distributed into three groups and received vehicle, RENCA cell vaccine, or RENCA Foxp3 cell vaccine for three weeks before they were inoculated with RENCA orthotopically. The tumor growth was monitored with Xenogen bioluminescence imaging and samples were collected when the study was ended around four weeks after inoculation. Excitingly, we demonstrated that both RENCA cell and RENCA Foxp3 cell vaccine significantly inhibited tumor growth in a pre-vaccine schedule, as compared to vehicle group (Fig. 2). Luminescence imaging shows that on day 7 after inoculation, 9 out of 10 vehicle mice had detectable tumor growth signals, whereas in the RENCA vaccine and RENCA Foxp3 vaccine treated group, only 1 out of 10 mice had detectable signals (Fig, 2B). On day 15 and day 22 after inoculation, the vaccine treated groups had dramatically lower luciferase activity (representing active tumor growth), as compared to vehicle group (Fig. 2A and B). At the end of study, both vaccine treated groups had significantly lower tumor weights than vehicle group (Fig.2C). It is noticeable that some mouse from vaccine treated groups had tumors with size comparable to those in vehicle group. This indicates that the vaccine may not induced anti-tumor immune response in these mice. Therefore, the effect of vaccine is not uniform in the same group.

Moreover, the result shown suggests tumor cell vaccine may be a robust immune therapy strategy in the RENCA model in a pre-vaccine schedule, which support testing of tumor cell vaccine for RCC in clinical setting. However, in this pre-vaccine schedule, the RENCA Foxp3 vaccine did not have additional anti-tumor activity when compared to RENCA cell vaccine. This is likely due to the fact that RENCA tumor cell vaccine is very immunogenic (in this schedule) and already very efficient to inhibit tumor growth. The possible effect on immunosuppressive cells may not appear additional benefit.

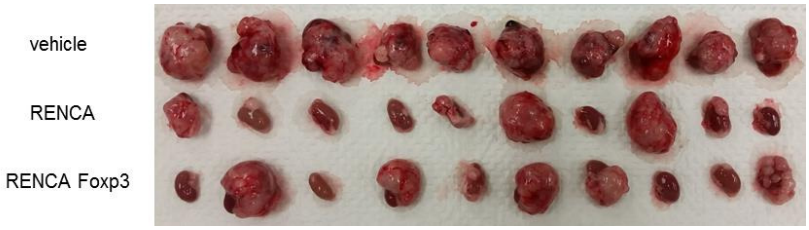
A


RENCA Foxp3 cell vaccine
RENCA cell vaccine
vehicle
Luciferase activity (ROI)
4×10⁶
3×10⁶
2×10⁶
1×10⁶
0
7
15
22
days after inoculation

B


Day 7
Day 15
Day 22
vehicle
RENCA
RENCA Foxp3

C


vehicle
RENCA
RENCA Foxp3

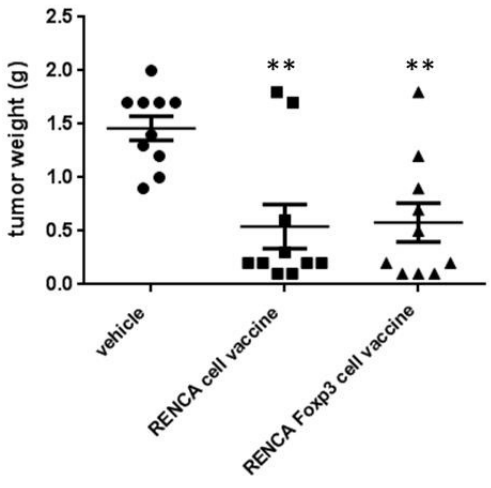

tumor weight (g)
2.5
2.0
1.5
1.0
0.5
0.0
**
**
vehicle
RENCA cell vaccine
RENCA Foxp3 cell vaccine

Figure 2: Effect of tumor cell vaccine in a pre-vaccine schedule. Female Balb/C mice were randomly divided into three groups and received vehicle, RENCA, or RENCA Foxp3 tumor cell vaccine for three weeks, then inoculated with RENCA cells in the sub-capsular space of the right kidney. **A.** plot shows tumor growth accessed by luminescence imaging. Tumor growth is represented by luciferase activity. **B**. pictures show the image of three treatment groups taken at different time points. **C**. top panel shows picture of tumors harvested from all groups at the end of treatment. Bottom panels show the end of experiment tumor weights. ** $p< 0.01$

In parallel, we have tested the vaccines in two more schedules with vaccine treatment started at day 2 or 7 days after inoculation, respectively (Figure 3 and 4).

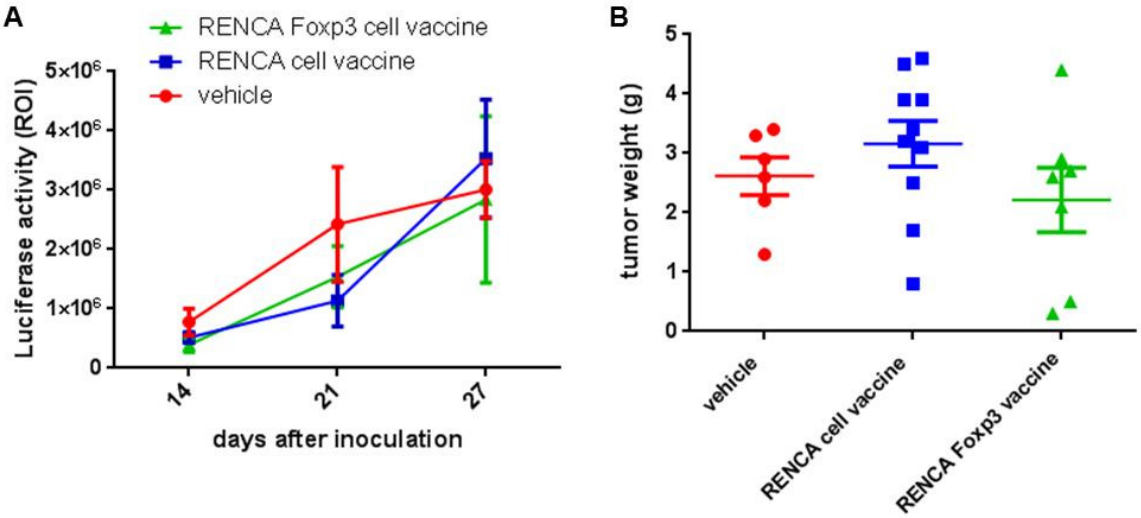


Figure 3. Effect of tumor cell vaccine with start of the treatment at the day after inoculation (day 2). Female Balb/C mice were inoculated with RENCA cells in the sub-capsular space of the right kidney. Next day, the mice were randomly divided into three groups and received vehicle, RENCA, or RENCA Foxp3 tumor cell vaccine. **A.** plot shows tumor growth accessed by luminescence imaging. B. End of experiment tumor weights.

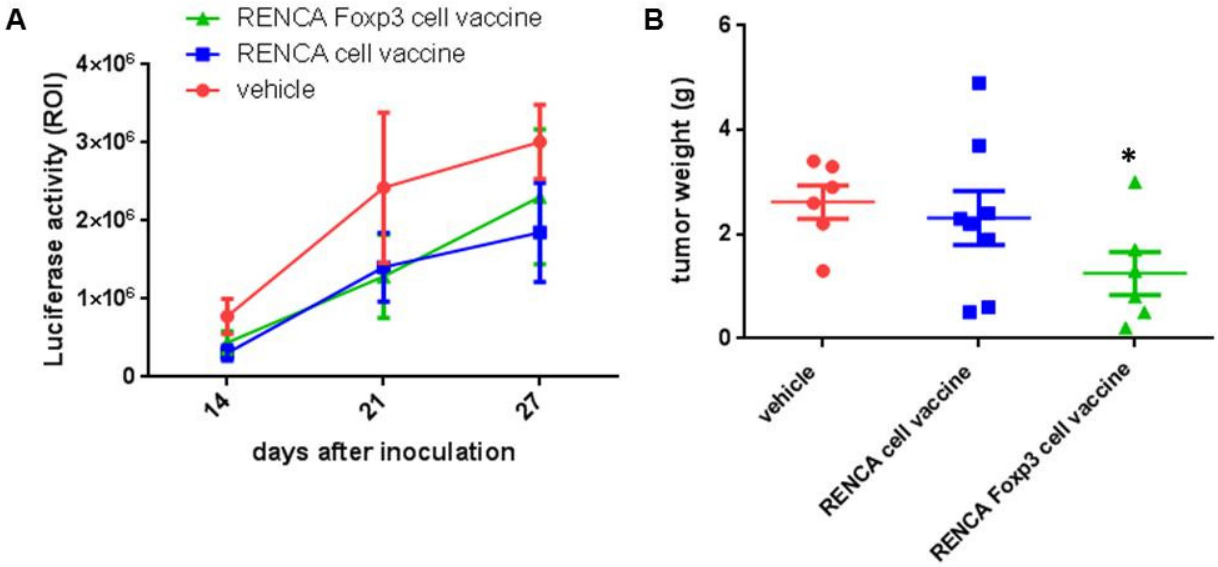


Figure 4. Effect of tumor cell vaccine with start of the treatment 7 days after inoculation. Female Balb/C mice were inoculated with RENCA cells in the sub-capsular space of the right kidney. 7 days after inoculation, establishment of tumor growth was confirmed with Xenogen bioluminescence imaging. Then the mice were divided into three groups so mice in each group had similar distribution of tumor growth. The three groups received vehicle, RENCA, or RENCA Foxp3 tumor cell vaccine. **A.** plot shows tumor growth accessed by luminescence imaging. **B.** End of experiment tumor weights. * $p<0.05$

In a schedule when vaccine started 1 day after inoculation (day 2), neither RENCA nor RENCA Foxp3 tumor cell vaccine had significant antitumor activity (Figure 3). At day 21, vaccine treatment groups had a trend to have lower luciferase signal, as compared to vehicle group (Figure 3A), but the difference was not significant. By day 27, the moderate difference disappeared. It has been noticed that, sometime when tumor grew big, less efficient of perfusion of tumor by the luciferin reagent may cause low/dropped luciferase activity even with a very big tumor size by palpation. Therefore, when tumor grows big, the luciferase signal from luminescence imaging is not reliable to represent the tumor size anymore. In this case, we rely on the end of treatment tumor weights to access the anti-tumor activity of the treatments.

In this schedule, the vaccine treatments did not make a difference on the tumor weights either. Based on our experience, the RENCA model carried in our lab is more aggressive recently than it was before. We use to inject 0.5 million RENCA cells, the tumor growth usually were detectable 12 days after inoculation. For this experiment, we injected only 10,000 cells and substantial signals were detected with a week. It is possible that the model is very aggressive tumor model and there was not enough time window for the vaccine to be effective to induce anti-tumor response, especially aggressive tumor growth can quickly induce immunosuppressive macro- and microenvironment to inhibit anti-tumor immune response and impair the vaccine effect. More discussion will follow based on assessment of tumor microenvironment. To improve this experiment, we will inject even less cells to start tumor growth to allow more time for vaccine treatment or skip the matrigel in the inoculates to make tumor growth less aggressive.

Experiment with a schedule when vaccine started 7 day arise a very interesting result (Figure 4). Although the bioluminescence imaging assay did not show difference of tumor growth between groups, RENCA Foxp3 tumor cell vaccine treated group, not RENCA tumor cell vaccine treated group, had significantly lower tumor weight, as compared to vehicle group. During the Xenogen imaging process, we did notice that in vehicle and RENCA tumor cell vaccine groups, there were big tumors with little signal, probably due to poor perfusion in a big tumor, which might have caused a drop of the increase rate of the signal from day 21 to 27 (Figure 4A). Therefore, here we based our analysis on the tumor weight result. It is not fully understood why the RENCA Foxp3 vaccine was superior to RENCA vaccine in a schedule with start of treatment 7 days after inoculation, but not in a schedule with start of treatment the day after inoculation. One possibility is that the GM-CSF in vaccine doses may facilitate accumulation of suppressive myeloid cells and establishment of tumor growth, when applied upon inoculation of tumor cells. We will further investigate the mechanism underline this phenomenon as planned in the Specific Aim 3 during the next period.

Clinically, tumor cell vaccine is for RCC patients with diagnosed disease, which resemble the third schedule when treatment given to animals with established tumors. The result that RENCA Foxp3 tumor cell vaccine had significant anti-tumor activity supports and provides reference for test the similar tumor cell vaccine in clinical setting for RCC patients.

For the correlative study, we have done extensive analysis to evaluate the components in peripheral immune environment and tumor immune microenvironment. We performed immunofluorescence staining and flow cytometry to access the composition of immune cells in the peripheral sites at different time points and in the tumor infiltrates (tumor microenvironment). Test of CD4 cells, CD8 cells, Tregs, myeloid cell populations

including MDSCs macrophages, CD11c dendritic cells have been done. Here we have a figure (Figure 5) contains panels only with representative analysis results. Analysis of effect on Tregs, MDSCs macrophages, and other myeloid cells are summarized in the following accomplishment under Specific Aim 3.

Based on these analyses, the tumor cell vaccine did not induce significant change in composition of T lymphocytes (CD4 and CD8) in blood and in tumor infiltrating immune cells (Fig.5A, B, and C). One result really stands out is that RENCA Foxp3 vaccine, not the RENCA cell vaccine, dramatically induce lymphocyte infiltration in the tumors (Fig. 5D). This is very interesting as possible increase of effector lymphocytes in the tumor may correlate with the anti-tumor activity of the treatment. We will further investigate this finding and underlying mechanism during the next period.

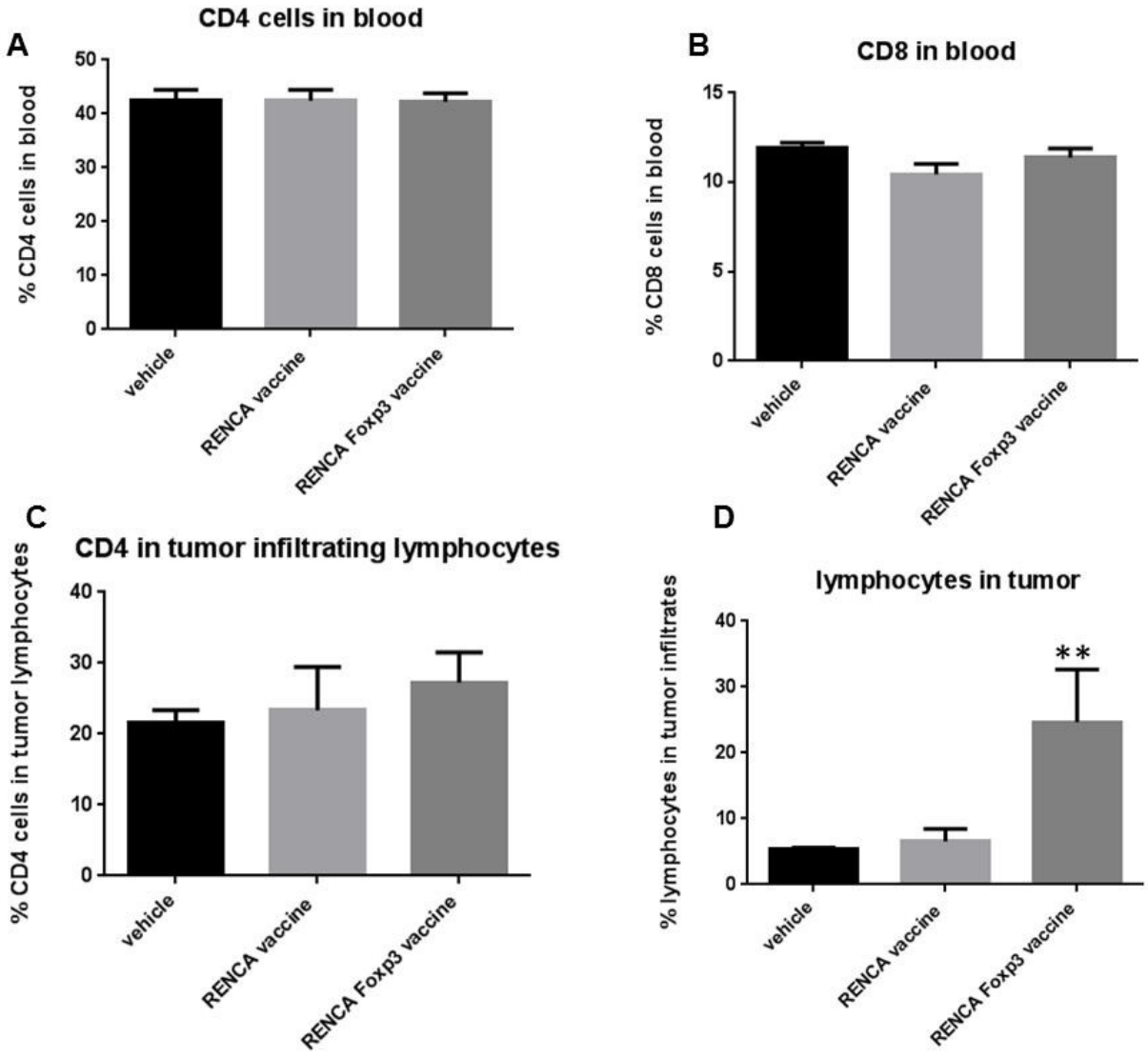


Figure 5. Effect of tumor cell vaccine on lymphocytes populations. Blood samples and tumor suspension samples were prepared from differentially treated mice and subject to immunofluorescence staining and FACS analysis. A. percentage of CD4 cells in blood. B. percentage of CD8 cells in blood. C. percentage of CD4 cells in tumor infiltrating lymphocytes. D. percentage of lymphocytes in tumor infiltrates. $^{**}p<0.01$

Task 6: Conduct survival studies with RENCA Foxp3 cell derived vaccines and the control vaccines. (Month 10-14).
We will accomplish this task during next period as planned.

There is a delay on performing the vaccine study in form of dendritic cells (DCs). We tried to improve the RENCA model to be a metastatic model. Usually, mice will die from primary tumor before lung metastasis develops. We have been trying nephrectomy procedure to resect orthotopic tumor to allow mice survive to develop lung metastasis. We'd like to perform the vaccine therapy in form of DC in the nephrectomy, metastatic model to better simulate clinical situation when patients have surgery and look for a strategy to inhibit metastasis disease. However, the development of the nephrectomy, metastatic mode took long time. We have made progress on survival of the animal from nephrectomy and delaying recurrent tumor on the kidney site, but the model is still not ready for larger scale study. So now we are keeping the original plan, and the vaccine therapy in form of DC is now ongoing in current standard RENCA model.

Specific Aim #2: To determine the anti-tumor activity of tasquinimod in combination with the dual-targeting vaccines in the RENCA model. (Month 10-19)

Task 1: Conduct therapy experiment to test the combination of the RENCA Foxp3 cell derived vaccines and tasquinimod. (Month 10-15)
Task 2: Correlated studies. (Month 10-17)
Task 3: Data collection and statistical analysis. (Month 15-17)
Task 4: Conduct survival studies to test the combination of the RENCA Foxp3 cell derived vaccines and tasquinimod. (Month 15-19)

We will be focusing on completing the tasks under Specific Aim 2 during the next period as planned.

Specific Aim #3: To assess tumor-specific immune response after the delivery of the dual-targeting vaccine alone or in combination with tasquinimod. (Month 6-24)

Task 1: Assess tumor-specific responses in therapy experiments. (Test after dual targeting vaccine single therapy: Month 10-14; test after combination studies: Month 15-19)

Task 2: Evaluate the effect of treatments on immunosuppressive cell populations and other immune cell populations. **(Evaluation of dual targeting vaccine single therapy: Month 6-10**; evaluation of combination studies: Month 11-15)

Task 3: Study the mechanisms by which tasquinimod suppresses MDSCs, and search additional molecular targets. (Month 17-24)

Task 4: Data collection and statistical analysis. (Month 8-24)

Here we summarized accomplishment under Specific Aim 3, Task 2: Evaluate the effect of treatment on immunosuppressive cells populations and other immune cell populations.

First we analyzed the effect of vaccine treatment on the blood Tregs. In the pre-vaccine schedule, RENCA Foxp3 vaccine treatment did not change the numbers of the Tregs (Fig. 6A). Interestingly, the RENCA Foxp3 tumor cell vaccine did significantly reduced Foxp3 expression level in Tregs, shown as reduced mean

fluorescence intensity of Foxp3 staining (Fig. 6B and C), as compared to vehicle and RENCA cell vaccine group. In other two treatment schedule, RENCA Foxp3 cell vaccine did not have the same effect on blood Tregs.

Another finding is that RENCA Foxp3 cell vaccine significantly reduced Tregs number in tumor infiltrates as well as Foxp3 expression level in Tregs (Fig.7A and B). Again, we did not observe the same in other treatment schedules.

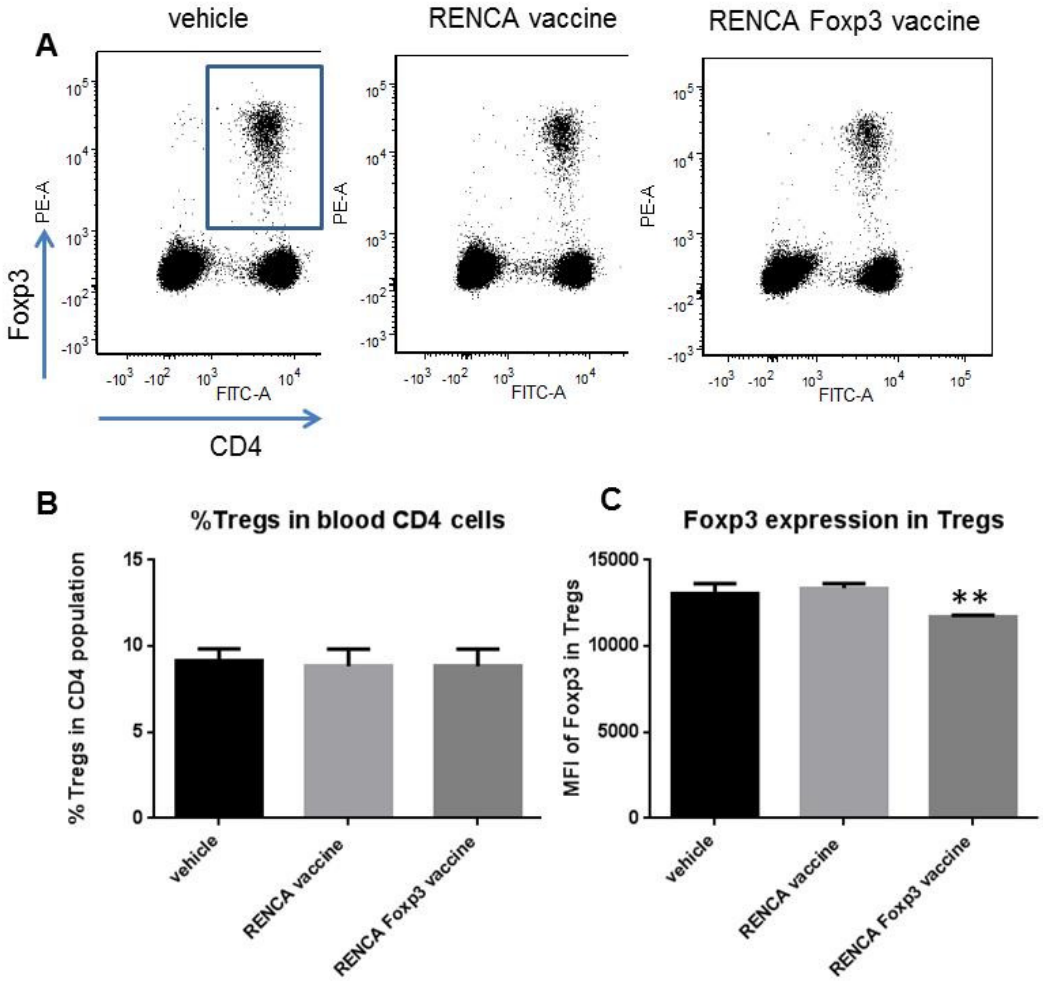


Figure 6. Effect of tumor cell vaccine on blood Tregs in pre-vaccine schedule. Blood samples obtained from differentially treated mice and subject to immunofluorescence staining and FACS analysis. A. flow cytometry plots of Tregs analysis. B. percentage of Tregs cells in blood CD4 cells. C. Mean fluorescence intensity of Foxp3 staining. *p<0.05

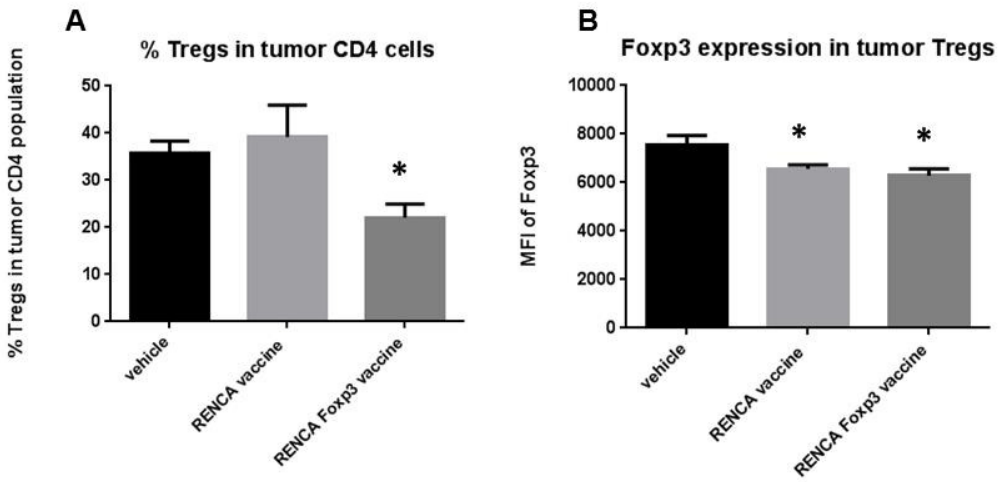


Figure 7. Effect of tumor cell vaccine on tumor infiltrating Tregs in pre-vaccine schedule. Tumor suspension samples were prepared from differentially treated mice and subject to immunofluorescence staining and FACS analysis. A. percentage of Tregs cells in tumor infiltrating CD4 cells. C. Mean fluorescence intensity of Foxp3 staining. *p<0.05

Besides Tregs, we have also evaluated myeloid immunosuppressive populations in blood and in tumor microenvironment. Here we present the results from tumor suspension samples. Based on these analysis, orthotopic RENCA tumor is a tumor model with extensive accumulation of immunosuppressive myeloid cells such as myeloid derived suppressor cells (MDSCs) (Fig. 8A) with Ly6G positive granulocytic MDSCs as dominant population (Fig. 8B), and M2 polarized tumor associated macrophages (TAMs). The vaccine treatments did not have any effect on these myeloid suppressor cells. The accumulation of these myeloid suppressor cells in the tumor maintain the immunosuppressive tumor microenvironment, and can block the induction of immune response against tumor antigen as well as against Foxp3 positive Tregs. Therefore, we need to target these cells while we target tumor antigen and Foxp3 with vaccine strategy in the RCC model, which is planned in the next period of the award.

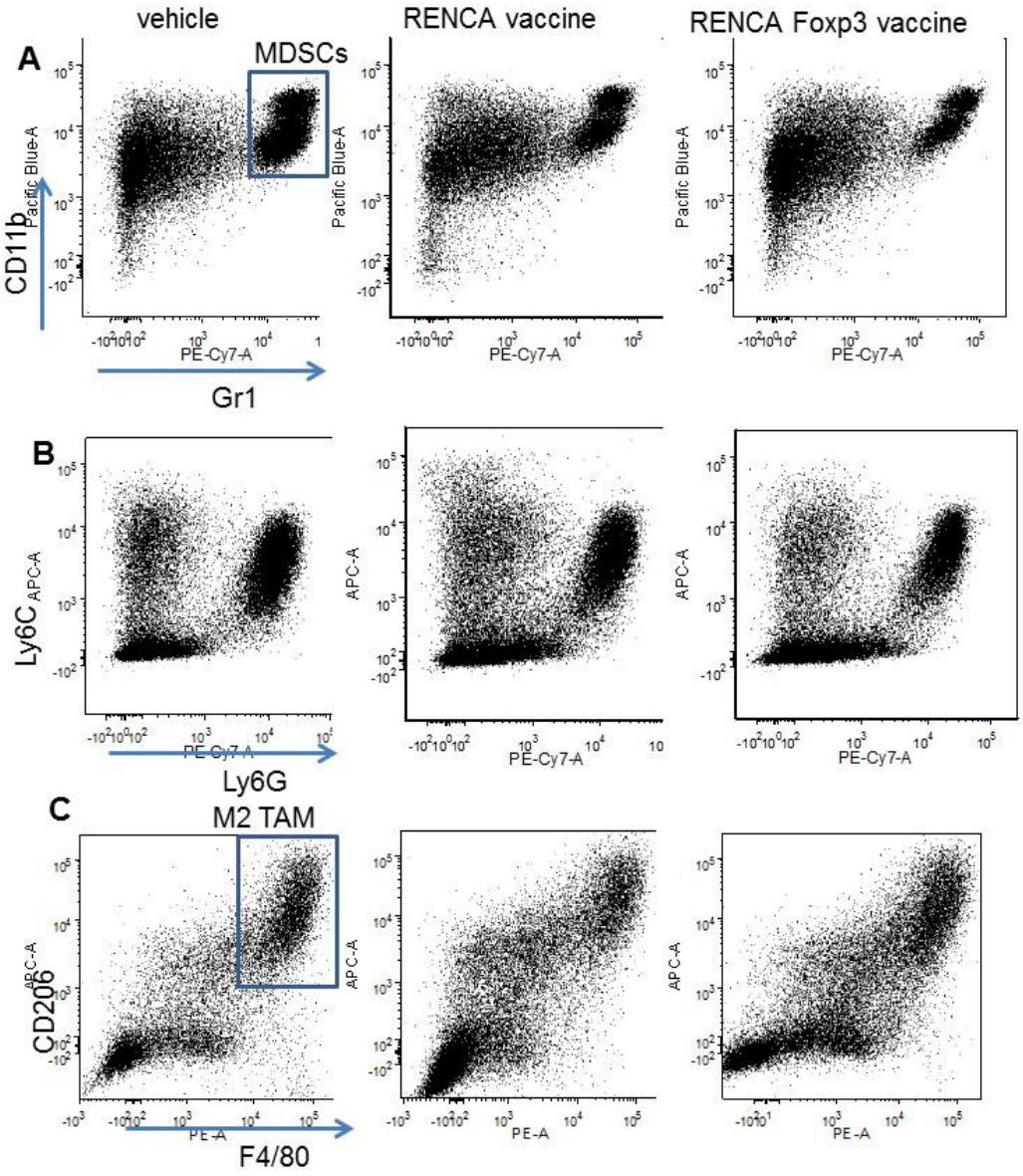


Figure 8. Suppressive myeloid cells accumulated in the RENCA tumor and can be obstacle of vaccine strategy. Tumor suspension samples were prepared from differentially treated mice and subject to immunofluorescence staining and FACS analysis. A. MDSCs in tumor. B. Granulocytic MDSCs are the dominant MDSCs in RENCA tumors. C. M2 polarized TAMs in RENCA tumors.

Impact:

Our results show that tumor cell vaccines, both RENCA cell vaccine and RENCA Foxp3 cell vaccine, successfully prevent the tumor growth from a very aggressive, orthotopic murine RCC model. In addition, The RENCA Foxp3 tumor cell vaccine is superior to the RENCA tumor cell vaccine, and had significant anti-tumor activity in an intervention setting of experiment. Moreover, RENCA Foxp3 tumor cell vaccine had activity to reduced number of tumor infiltrating Tregs and to reduce Foxp3 expression level inn Tregs in blood and tumor infiltrates. The accomplishment and finding from this period are exciting, because they support test of tumor cell vaccine in RCC in clinical setting. Especially, the result suggest a strategy target Tregs with vaccine therapy or a dual targeting vaccine strategy may be beneficial and may have higher success rate in treating patients with establish disease. The dual targeting vaccine strategy is also expected to be a candidate therapy for patients who have surgery to prevent recurrent and metastatic disease.

One thing we also learned from this period is that the myeloid suppressor cell populations are big obstacle to an efficient vaccine strategy. Our result show much dramatic anti-tumor effect of vaccines in a prevention schedule, as compared to intervention schedule. This is very likely because in animal with established tumor an immunosuppressive environment is already established, and one of the important immunosuppressive factors is myeloid suppressor cell populations. Target these myeloid suppressor population will be critical to success of the dual targeting vaccine strategy, which is planned in our next period of the project.

Changes: nothing to report**.**

Products: The RENCA Foxp3 cells with Foxp3 over-expression as source of the dual-targeting vaccine.

Participant:

Name: Li Shen;
Project Role: PI;
Person month worked: 10.8
Contribution to Project: Dr. Li Shen has performed most of the experiment of the project and conduct data analysis.
Funding support:

Special Reporting Requirements: non-applicable**.**

Appendices: Nothing to report.